Silk Fibroin-Based Scaffolds: Potential for Applications in Wound Healing

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Abstract

Chronic wounds, such as diabetic foot ulcers, pose significant challenges due to their prolonged inflammation state, slow vascularization, lack of supportive matrix, and frequent recurrence. Biocompatible materials like silk fibroin (SF) and collagen can be promising in chronic wound healing applications due to their biocompatibility, and ability to act as supporting matrix necessary for tissue regeneration. This study explores the development and characterization of SF-based biomaterials in various formats, including gels, lyophilized scaffolds, and films, by combining them with collagen (Col) and heparin (Hep) molecules that are known for the wound-healing properties. SF gels, films, and lyophilized scaffolds were characterized for water retention capacity, porosity, contact angle, and antibacterial properties. Curcumin-loaded films reported moderate wetting behavior but were brittle, thin and hard. Freeze-dried scaffolds possessed higher water retention capacity compared to the sonicated gels and films, and showed a porous network structure, while the fabricated SF gels disintegrated in water. SF with a chelating agent demonstrated mild antibacterial properties, which were retained when combined with Collagen peptides and Heparin. The antimicrobial effect was significantly enhanced when SF+Col+Hep was added with antimicrobial agents such as Gentamicin and Ceftriaxone. The study concludes that SF-based scaffolds, particularly those incorporating collagen peptides and heparin, can be leveraged for molecular delivery application towards wound healing. Future research should focus on improving mechanical properties, measuring release kinetics of heparin and other biomolecules from SF-based lyophilized scaffolds, and conducting in vivo studies to validate the therapeutic value.

Keywords: Silk fibroin, collagen, heparin, antimicrobial agents, wound healing.

1. Introduction

Chronic wounds affect millions of people globally, posing significant emotional and economic burdens. Chronic wounds, such as diabetic ulcers, venous ulcers, and pressure sores, often require advanced materials that can provide a conducive environment for healing[1]. Current

treatment options often fall short due to inadequate vascularization, moisture retention capacity, and weak mechanical properties [2]. To ensure that every aspect of chronic wound healing is addressed, ideal biomaterials should be capable of providing strong mechanical support, being able to retain moisture, showing antibacterial effects, and delivering molecular entities such as vascularization-inducing agents, growth factors, etc.[3].

Silk fibroin (SF) and Collagen (Col) are two promising biocompatible proteins with significant potential for wound healing. SF is known for its mechanical strength, biocompatibility, and biodegradability and also helps in cell migration, proliferation, and differentiation while encouraging the growth of new tissue close to the wound [4], while collagen is a major component of the extracellular matrix (ECM), supporting cell growth and tissue integration [5]. Combining these materials with Heparin (Hep), which has an affinity for vascular endothelial growth factors (VEGF), could create a synergistic effect, enhancing vascularization and tissue regeneration[6]. In addition, molecules such as Curcumin known for its anti-inflammatory properties can also be added to the SF based natural biomaterials, to help in reducing swelling by actively participating in NF-κB pathway, thus enhancing healing [7]. Antibiotics like Gentamicin and Ceftriaxone can also be explored for delivery with SF as both antibiotics contribute significantly to wound healing by preventing and treating local and systemic infections, reducing inflammation and bacterial load leading to faster healing[8].

While a lot about SF and Collagen has been explored, the literature is less for the functionalization of SF with biomolecules like Heparin in which preserving the native structure of protein without alteration is still not possible. Heparin accelerates wound healing through multiple mechanisms like the promotion of angiogenesis, fibroblast proliferation and collagen synthesis and protection of growth factors [9]. We wanted to explore methods of fabrication where SF matrices that can incorporate heparin with other biomolecules is possible for therapeutic applications such as tissue regeneration.

The objective of this study is to create SF-based matrices capable of holding Heparin, Collagen, Curcumin, and antibiotics together and to characterize these formats water retention, porosity, hydrophilicity, and antibacterial effect. To incorporate these, we used different techniques such as ultrasonication gel forming technique, lyophilization and film forming. Further we evaluated the antibacterial properties, and overall effectiveness of SF based materials.

The results are expected to demonstrate the potential of SF-based materials in addressing the critical challenges in chronic wound healing, specifically porosity and water retention results can inform studies for creating a conducive environment for cell proliferation and tissue repair.

2. Materials and Methods

Silk Fibroin (SF)

A 5% w/w solution of silk fibroin was obtained from Fibroheal Woundcare Pvt. Ltd. The obtained solution was aqueous 100% silk fibroin protein derived from Bombyx mori and contained a chelating agent as a preservative.

The product was manufactured in a controlled environment to minimize contamination.

Type I collagen peptides (Col)

Type I collagen peptides were obtained in kind from Dr. Madhan's laboratory, Central Leather Research Institute, India. The type I collagen peptides were formulated by them as described in their previous study[10]. The tendons were first dissected to remove any extraneous matter, then cut into smaller pieces and minced with crushed ice. The matrix was gelatinized by heating at 90 °C for 1 hour and then cooled to room temperature. Enzyme digestion was performed using trypsin at 37 °C for 3 hours. The digested product was filtered, and the filtrate was heated to 90 °C, cooled again, and finally lyophilized to obtain Type I collagen peptides.

Heparin (Hep)

Heparin Sodium Salt from the Hog intestine, Product H0393 was procured from Tokyo Chemical Industry Co., Ltd. (TCI), India. Four groups of solutions were prepared with varying heparin concentrations (0, 1, 10, and 100 μg/ml). These solutions are then mixed with the SF solution to form the hybrid bio grafts.

Antibiotics

Two antibiotics Gentamicin Sulfate and Ceftriaxone Sodium were purchased from Yarrow Chem products and Aayan Labs respectively.

Curcumin

Curcumin (diferuloylmethane) was obtained from SDFC Ltd.

3. Method

1. Formation of SF Films

A refined dialyzed SF solution in a pure form of 5% w/w was used to cast the films and another group was the films added with 50 mg of curcumin to 15 ml of SF with continuous stirring. The solution was then evenly poured onto flat glass petri dishes. During the film's drying phase, controlled conditions were maintained to avoid uneven film formation. The films were then dried for 48 hours at 40°C in a hot air oven (NSW 143 OSA-4) per method in another study [11].

2. Formation of Gels

For the formation gel, 3 different sonication conditions were followed are mentioned below:

Keeping the SF concentration constant as 5% w/w and varying the sonication time from 5-30 seconds at 40% amplitude, N=3; each gel sample was created on Sonicator (Cole Parmer-CPX500) . After sonication the samples were kept at 37°C for incubation and visual observations were noted regarding gelation, color, and flowability.

Further the same process was repeated to verify the effects of sonication on SF with Col, Hep and antibiotics. 2 ml sample of each of these groups was sonicated for 1 minute at 40% amplitude followed by same incubation conditions.

3. Formation of freeze-dried scaffold

The fibroin solution was frozen at -20ºC for 24 hours and then lyophilized to obtain silk fibroin sponge[12]. A pressure 0.016 mbar and temperature -50ºC was used. Labconco lyophilizer (Free Zone 2.5 Liter -50C Benchtop Freeze Dryer) was used to perform freeze drying. After freeze-drying for 48 hours, the sponges were formed, where different groups of SF alone, SF and Col (5mg/ml), SF with Col (5mg/ml) and Hep (50µg/ml), SF with Col (5mg/ml) and Hep (50µg/ml) and antibiotics (5mg/ml), with the quantity of 5ml each solution was used.

4. Physical Appearance by Optical Microscopy

Nikon Optical microscope (Model- H600L)) was used to capture the images of SF-based materials in different forms. A magnification of 10X was used. A stereomicroscope (Model C-LEDS 100-240V) was also used for the evaluation of scaffold samples.

5. Contact Angle Measurement

The contact angles of these films were measured using a Contact Angle Meter (Holmarc-HTCAM 103 230V/50HZ) by the water drop method, with a sample size of N=3. This method was used to calculate the contact angles of curcumin-loaded SF films to assess their wettability. Both left and right angles were considered, and the average indicated moderate wetting behavior. All experiments were performed twice (N=2).

Also, the same test was carried out for SF sonicated gels and freeze-dried scaffolds, however the water droplet was absorbed quickly and hence the contact angle calculation was not possible for these samples.

6. Water Retention and Porosity

Water retention was determined for the SF films, sonicated gels and freeze-dried samples. Water absorption was determined as the weight of the gel increased after absorbing water as mentioned in study by Kaplan [13]. For this, the dry gel with weight noted as W_1 was immersed in Millipore water which was filtered from 0.2µm membrane at 37°C for 10 min. Excess water was allowed to drip out, and the weight W_2 of the wet gel was measured again. The water absorption of the gel was calculated based on the following equation: water absorption $(W_2 - W_1)/W_1$. This experiment was performed for (N=3).

Furthermore, the porosity of the freeze-dried sponges were measured by the Liquid displacement method [14]. Ethanol was used because ethanol is capable of penetrating into the pores easily but do not cause size shrinkage or swelling to the material being tested. In brief, the scaffold was placed in a graduated cylinder with a known volume of the displacement liquid for 5 minutes. This is an indirect way of measuring the scaffold porosity. The open porosity was calculated using the equation $(V_1 – V_3)/(V_2 - V_3)$ where V_1= known volume of liquid that is used to submerge the scaffold, V_2= volume of the liquid and liquid-impregnated scaffold, and V_3= remaining liquid volume when the liquid-impregnated scaffold is removed. This experiment was performed in triplicate (N= 3).

7. Antibacterial Assay

First, Mueller Hilton (MH) Agar was prepared by dissolving 14.9g of agar in 400 ml of distilled water and sterilizing it in a 500 ml flask. The agar was then poured onto plates with a diameter of 34 mm and incubated overnight in a laminar hood. Meanwhile, a Luria Broth (LB) solution was prepared by mixing 0.25g of broth in 10 ml of distilled water, covering, and autoclaving it for 15 minutes at 120°C. After cooling to room temperature, the LB Broth was inoculated with E. coli MTCC47 and shaken for 24 hours at 37°C and 125 rpm. Subsequently, 100 µl of the E. coli culture was spread on the agar plates. Wells were punctured in the agar plates under a laminar hood to load solutions of SF, collagen, heparin, and antibiotics. Each well received 100 µl of the respective groups mentioned below and the plates were incubated for 24 hours at 37°C.

The study evaluated the antibacterial effects of various groups: SF with a chelating agent added as a preservative (not disclosed due to proprietary concerns from Fibroheal Woundcare Private Ltd., India), SF combined with collagen, a combination of SF, collagen, and heparin, and a combination of SF, collagen, heparin, and two antibiotics (Gentamicin and Ceftriaxone Sodium) and last the last one was SF with curcumin. Two control groups were included: one with distilled water and another was antibiotics alone.

The results were observed the next day, comparing against a negative control of distilled water and a positive control using antibiotics. All experiments were performed in triplicate (N=3).

8. Statistical Analysis

The data were analyzed using one-way ANOVA to determine significant differences between means of various groups. Statistical significance was considered at $p<0.05$. A two-sample t test assuming equal variance was performed for comparing means of two groups using Microsoft Excel.

4. Results

Physical appearance

The sonicated SF-gels stayed intact when test samples were inverted in tubes and no color change was observed (Fig. 1a). However, a granular appearance was observed on optical microscope (Fig. 1 f).

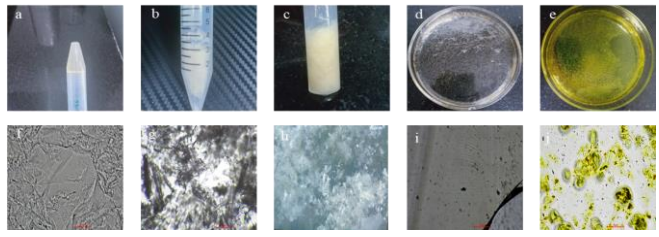

Fig. 1: Physical appearance of various sample formats of SF. a – Ultrasonicated gels, b- Lyophilized sponge, c- SF hydrated sponge d- SF films without curcumin, e – SF film with

curcumin; Images (f-j) are optical microscopic representations of following samples. f- sonicated gel at 50µm, g- lyophilized sponge at 50µm, h-stereomicroscope image of lyophilized sponge at 100 µm, i- SF films without curcumin at 50µm, j- SF films with curcumin at 50µm.

The freeze-dried sample showed a porous and network-like structure (Fig. 1 b). The optical and stereomicroscopic images indicated the formation of crystalline structure (Fig. 1 g). The image of lyophilized sponge when rehydrated with water is captured in (Fig. 1 c) and stereomicroscopic image of the rehydrated sample is shown in (Fig. 1 h). The yield calculated for the freeze-dried scaffold was 6.28%.

The SF film appears to have a transparent, relatively smooth, and uniform surface (Fig. 1 d) with some visible cracks and possibly aggregated regions (Fig.1 e). The curcumin-loaded SF films (Fig. 1 e) had a non-uniform formation and showed separate curcumin-containing pockets within the SF films (Fig. 1 j) as curcumin did not dissolve in the SF matrix during film formation. However, the SF films were very brittle and difficult to handle due to their thin layered structure.

Wetting behavior of films

The results for contact angle measurement for curcumin containing-SF films indicated moderate wetting behavior, with an average left angle of 51.3 ± 15.14 degrees and right angle of 52 ± 15.13 degrees, suggesting moderate hydrophilicity, which can be beneficial for cell adhesion and proliferation [15]. However, the SF films formed after drying were very brittle, and addition of curcumin gave very patchy and undissolved pockets within SF films, suggesting that further improvements are needed to enhance the solubility of curcumin within the SF.

Water Retention Capacity and Porosity

In the water retention study, it was observed that the sonicated SF gels were disintegrating and dissolving in the water, whereas the freeze-dried scaffolds retained their structures and absorbed the water. The scaffolds showed a significantly higher retention capacity percentage of 554 ± 233.56 (mean ± SD), whereas the sonicated gels comparatively had significantly lower values of 100.98 ± 78.66 56 (mean ± SD). Two-sample t test assuming equal variance performed for all groups confirmed a statistically significant difference ($p<0.05$) between mean water retention values for Sonicated gels vs. Freeze-dried (FD) scaffolds, for groups of SF, SF+Col, SF+Col+Hep, and SF+Col+Hep+Genta, as seen in Fig. 2.

The water retention % of SF films was compared against SF gels and SF scaffolds (not shown in Fig. 2). SF film water retention capacity % was 372 ± 34.82 (mean ± SD), which was higher than that of the sonicated gels but lower than the freeze dried scaffolds.

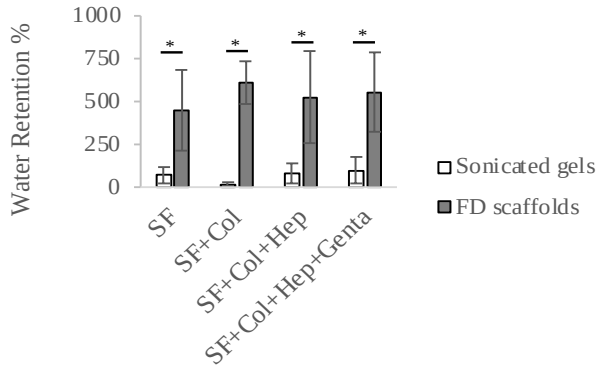

Fig 2: Bar graph represents the water retention % of SF alone and SF with biomolecules for gels and freeze-dried scaffolds. N=3 samples.

In porosity testing, scaffolds showed a significant hold of the structure while being immersed in ethanol and absorbed it completely, whereas the sonicated gels completely disintegrated and dissolved in the ethanol hence porosity test was not possible in the case of gels. The porosity value of scaffolds was found to be 79.51± 17.76 with a p-value of 0.48 between SF versus SF+Col, SF +Col+ Hep, SF+Col+Hep+Genta groups, indicating no significant difference in porosity occurred after addition of molecular entities such as collagen peptides, heparin or gentamicin.

Antibacterial Activity

The antibacterial assay against E. coli showed that SF with chelating agents alone and in combination with collagen and heparin maintained antibacterial activity. The addition of antibiotics like Gentamicin and Ceftriaxone further enhanced the antibacterial effect, as evidenced by the larger zones of inhibition in Fig. 3(a).

5. Discussion

This study explored the addition of Heparin, Collagen, Curcumin, Gentamicin, and Ceftriaxone within Silk Fibroin based biomaterials and characterized them in various formats of gel, lyophilized sponge, and films.

The water retention and porosity tests on scaffolds showed a significant hold of the structure while being immersed in water and ethanol respectively, and absorbed it completely, whereas the sonicated gels got disintegrated in the ethanol. This can be attributed to the weaker molecular arrangement of sonicated gels, compared to freeze-dried scaffolds, where slow cooling performed by ultrafreezing process is known to transform the amorphous SF form into crystalline β-sheets [17]. The β-sheet crystalline structure in the lyophilized scaffolds is probably induced by the physical cross-linking of SF during freezing and lyophilization processes. We observed that the freeze-dried scaffolds contained tiny crystals as observed in optical microscopy, which

needs to be confirmed using FTIR. The crystalline structure can be further enhanced by altering lyophilization time, process, etc. [16]. During the freezing step, solvent crystals form at a controllable rate, pushing solute molecules out of the frozen solvent until the sample is completely solidified. This step is essential for creating the desired porous structures, with the ability to control pore size and quantity based on variables like freezing temperature and rate [18].

SF Films were observed to be brittle, and although they had water retention capacity slightly less than the scaffolds, their biggest issue was inflexibility and hardness. The moderate wetting behavior of the dried SF films could be advantageous for cell adhesion and proliferation; however, given their patchy, thin layered nature and inhomogeneous pockets of curcumin observed, their application to wound healing was not found suitable in this work.

Overall, between sonicated gels and lyophilized scaffolds, the freeze-dried scaffolds formed a well-connected porous structures that retained mechanical integrity in water and offered a significantly enhanced water retention capacity compared to the gels. These lyophilized scaffolds deserve further exploration for potential to deliver biomolecules such as heparin to increase vascularization in chronic wounds by attracting vascular endothelial factor (VEGF) to wound site.

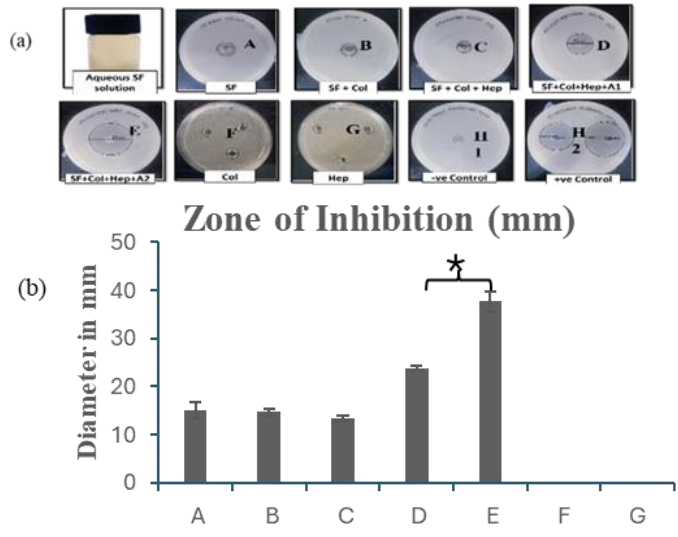

Fig. 3(a): Zone of inhibition for various groups tested against E. Coli bacteria: A=SF; B=SF+Col; C=SF+Col+Hep; D=SF+Col+Hep+Genta; E=SF+Col+Hep+Ceftri; F=Col ; G= Hep. H1= Negative control of distilled water, and H2 =5mg/ml antibiotics (Gentamicin alone, Ceftriaxone Sodium).3(b) Bar graphs represent the mean diameters of inhibition zones + std. dev for N=3 samples.

This study also showed that silk fibroin (SF) solution at a concentration of 5% w/w exhibited a 15 mm diameter zone of inhibition, indicating mild antibacterial activity on account of the

chelating agent in the SF solution (not disclosed due to proprietary concerns of Fibroheal company). The combinations of Silk Fibroin + chelating agent when added with other biological entities such as collagen, heparin, and both collagen and heparin demonstrated preservation of mild antibacterial effects (Fig. 3).

In contrast, collagen and heparin alone without a chelating agent did not produce any zone of inhibition (Fig. 3, F and G), like the negative control of water (Fig. 3 H1). The incorporation of antibiotics Gentamicin and Ceftriaxone further enhanced the zone of inhibitions significantly (Fig. 3 D and E), boosting the potential of the SF+Coll+Hep hybrid scaffolds for antibacterial drug delivery in wound-healing applications.

6. Conclusion

In summary, the results indicate that SF-based lyophilized scaffolds have excellent water retention capacity (mean + SD of 554 ± 233.56) as compared to sonicated gels (mean + SD of 100.98 ± 78.66). The, and porosity of SF-based lyophilized scaffolds was reported to be 79.51± 17.76, which can be beneficial for cell proliferation and vascularization needed for wound healing.

We found that SF-based materials can be combined with biological entities such as collagen, heparin, and antibiotics while retaining their porosity and water retention capacities. This holds significant promise for drug delivery and wound healing applications. Conversely, the SF films formed after drying were very brittle, suggesting that further improvements are needed to enhance their mechanical strength and flexibility. The study also demonstrated that SF-based materials when added with a chelating agent, demonstrate significant antibacterial properties against E. coli, that are preserved after addition of collagen and heparin entities. The addition of antibiotics Gentamicin and Ceftriaxone further enhanced these properties, increasing the potential of the hybrid scaffolds towards antibacterial drug delivery application for wound-healing.

7. Future scope

Future research will focus on optimizing the mechanical properties of the lyophilized scaffolds and conducting degradation and release kinetic studies to validate their effectiveness. Extensive in vivo studies are necessary to validate the efficacy and safety of these scaffolds in real-world wound-healing scenarios. Exploring the long-term stability and degradation behavior of SF scaffolds under physiological conditions will also be critical for their practical application in regenerative medicine. This continued research will be essential in advancing SF-based materials toward clinical use, offering promising solutions for chronic wound management and tissue engineering.

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